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PRINCIPAL INVESTIGATOR: Celine Aznar-Derunes, Ph.D.

CONTRACTING ORGANIZATION: The Burnham Institute  
La Jolla, California 92037

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**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**The Burnham Institute  
La Jolla, California 92037**8. PERFORMING ORGANIZATION  
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Mutations or changes in normal cellular genes are generally linked to human cancer. Multiple gene changes involving at least two types of cancer genes, protooncogenes and tumor suppressor genes, are required for the clonal expansion of a malignant cell. The RIZ gene plays an important role in human cancer and more particularly in breast cancer. RIZ is the founding member of the PR-domain family of zinc finger genes. Two protein products are produced from the RIZ gene which differ by the presence or the absence of the PR domain : RIZ1 and RIZ2. RIZ1 is commonly lost or underexpressed in tumors whereas RIZ2 is always present. RIZ1 is a tumor suppressor whereas RIZ2 is not.

In this project, we are trying to characterize the structural and the functional role of the RIZ1-PR and/or related domains using protein crystallography. Our goal is to understand the tumor-suppression mechanism of RIZ1 and PR-proteins in breast cancer. This will provide us new tools for possible treatment in breast cancer.

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## Table of Contents

|                                   |   |
|-----------------------------------|---|
| Cover.....                        | 1 |
| SF 298.....                       | 2 |
| Table of Contents.....            | 3 |
| Introduction.....                 | 4 |
| Body.....                         | 5 |
| Key Research Accomplishments..... | 8 |
| Reportable Outcomes.....          | 8 |
| Conclusions.....                  | 8 |
| Bibliography.....                 | 9 |
| Appendices.....                   |   |

## INTRODUCTION

The retinoblastoma interacting zing finger (RIZ) gene was isolated originally in a functional screen for proteins that bind to the retinoblastoma (Rb) tumor suppressor (1). RIZ maps to the human chromosome band 1p36.23 (2,3). This region commonly undergoes deletions, rearrangements or loss of heterozygosity in tumors including human mammary cancer (4).

RIZ is the founding member of the PR-domain family zinc finger genes. It contains an Rb-binding motif called the AR domain similar to the E1A viral oncoprotein (5), eight zinc finger motifs, some GTPase and SH3 domains and a PR domain at its N-terminus (6, 7, 8, 9). The RIZ gene normally produces two different protein products, RIZ1 and RIZ2, which respectively differ in length by the presence or the absence of the PR domain. RIZ2 is produced by an internal promoter (7). RIZ1 is a tumor suppressor whereas RIZ2 is not. RIZ1 expression is commonly lost in human breast cancer tumors as well as in several other types of tumor examined (9). In contrast, RIZ2 is uniformly expressed in all cases examined. So there may be a specific negative selection for RIZ1 versus RIZ2 in tumors.

We are focusing on the PR domain of RIZ1 which plays a role in tumorigenesis in breast cancer. The PR domain is composed of approximately 100 amino-acids and is conserved in a subfamily of kruppel-like zinc finger genes. Other members of the PR family include the MDS1-EVI1 breakpoint gene involved in human leukemia and the PRDI-BF1 or BLIMP1 transcription repressor, which can active B-cell maturation (10-13). Different observations suggest that PR genes or the PR-containing product of these genes are the negative regulators of cell growth and tumorigenesis. A common function of the PR domain is to mediate protein-protein interaction. The PR domain of RIZ1 is a protein-binding interface (6). Binding is mediated by residues conserved among different PR domains suggesting that similar functions may be shared among them.

The PR domain was found to be homologous to the SET domains (6) which have recently been shown to mediate protein-protein interaction too (14, 15). PR and SET domains are important in tumor suppression and define a new class of tumor suppressor genes (16). Furthermore, PR proteins may function in chromatin-mediated control of gene expression as inferred from the homology with SET proteins (6).

Our long term goal is to determine on one hand the crystal structure of the RIZ1-PR domain and on the other hand the crystal structure of some related PR domains: the BLIMP-PR domain called BLPR and one part of the entire RIZ1 protein containing the PR and AR domains. Doing this, we will understand the tumor-suppression mechanism of RIZ1 and PR-proteins in breast cancer.

Now, we have successfully produced milligram amounts of all these 3 proteins and they have been introduced into crystallization trials in order to solve their structure.

## **BODY**

### **Task 1 : To construct a stable RIZ PR domain.**

This task was already accomplished in our last report.

### **Task 2 : To crystallize the RIZ PR domain.**

#### *a) Crystallization trials :*

In our last report we started crystallization trials on the RIZ-PR domain in our lab by using pre-made crystallization kits from Hampton-Research (Crystal screens I and II) and Emerald Biostructure (Wizard I and II) or our own crystallization systems (changing temperature, concentration...). We sent too a sample to a facility for high throughput screening (Buffalo, NY).

These crystallization trials were done on the RIZ-PR domain alone, or in presence of co-factors : the S-adenosylmethionine (SAM) and the S-adenosylhomocysteine (AdoHcy) (17-22). Up to this time we have not be able to detect any interesting crystals.

But the length of this construct (163 amino-acids) allowed us to try NMR experiments. As the first results were very promising, our colleague the Dr Klara Briknarova is continuing these experiments to find the NMR structure of this PR domain.

### **Task 3 : To solve the crystal structure of RIZ PR domain.**

This part depends on the task 2, so we did not begin this part.

### **Task 4 : To solve the crystal structure of related PR domains.**

The main part of our efforts were concentrated on this task.

#### *a) Protein expression, purification, characterization*

- BLIMP-PR called BLPR

This part was already accomplished in our last report.

- RIZ1 protein

***RIZ1 sequence:***

**<sup>1</sup>GSPMDQNTTEPVAATETLAEVPEHVLRLPEEVRLFPSAVD~~K~~TRIGVVATKPKLKGGKEFG  
 FFVGDKKKRSQVKNNVYMWEVYYFNLGWMCHDATDPEKGNWLRVYNWACSGEEQNL  
 FPLEINRAIYYKTLKPIAPGEELLVWYNGEDNFEIAAAIEEEERASAR~~R~~KKRSSPKSRKGKKK  
 SQENKNKGNGKIQDIQLKTSEPDFTSANMRDSAEGPKEDEEKPSASALEQPATLQEVASQE  
 VPPELATPAPAWEPQPEPDERLEAAACEVNDLGEEEEEEEEEEEEEEEEEDDDDDDELEDEGE  
 EEASMPNENSVKEPEIRCDEKPEDLLEE<sup>325</sup>**

In our last report we talked about the difficulties encountered during the RIZ1 protein purification because of the degradation of the protein during the process. Last time, we determined by mass spectroscopy and amino-acid analysis that the degradation problem occurred between the 2 independently folded domains of RIZ1: PR (in red) and AR (in green). The only arginine present in this region is the arginine 160. To prevent tryptic-like cleavage, Dr Shi Huang, our collaborator, prepared a GST-fusion RIZ1 mutated the arginine 160 to alanine.

This mutant protein product is more stable. We see no degradation during the purification process which consists of : an affinity chromatography (Glutathione-Agarose column), thrombin cleavage followed by ion-exchange chromatography (Q-Sepharose column) to remove the GST-partner and then hydrophobic interaction chromatography (Phenyl-Sepharose column).

Activity experiments done after purification showed that the activity of RIZ1 concerning the methylation of the Histone 3 was retained after the mutation.

Unfortunately, stability experiments done with the purified protein showed that this protein was not stable at room temperature after concentration at 10mg/ml, even in the presence of a protease inhibitor like the 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF).

Therefore, additional protocols were developed to avoid this problem. The protein is stabilized by the following treatment:

- all the purification steps have to be done in presence of the protease inhibitor phenylmethylsulfonyl fluoride (PMSF)
- after all the purification steps listed above a gel filtration (Superdex-75 column) is required to remove traces of protease.
- the protein is unfolded in 8M urea in presence of 2mM EDTA and refolded by decreasing step by step the quantity of urea (4M, 2M and 0M urea) in presence of 2mM MgCl<sub>2</sub>.
- ion-exchange chromatography (Q-Sepharose column) is used to separate non-folded protein from the refolded protein.
- the protein is concentrated in a buffer containing 25mM glycine (pH 7.2), 1mM AEBSF, to 20mg/ml.

*b) Crystallization trials*

- BLIMP-PR called BLPR

The PR domain of BLIMP was introduced into crystallization trials with and without the two co-factors SAM and AdoHcy. Screens were performed with the Hampton Research reagents and the Emerald Biostructure kits and more particularly the conditions which contain a high salt concentration which were used for homologous SET domains. Samples were sent to a high-throughput screening facility also.

From the crystallization screens, to two sets of conditions are promising :

- 8% Jeffamine, 1.15M ammonium sulfate in which we obtained tiny crystals
- 20% PEG 8000, 0.05M potassium phosphate in which we obtained needle clusters

So far crystals that are suitable for diffraction have not been produced.

Now we are planning to produce with the help of our collaborator Dr Huang a shorter BLPR construct. We will try to trim the terminal regions of the domain that we expect may be misfolded or flexible, to produce a more compact domain.

- RIZ1 protein

Right now, as we have the stable RIZ1 protein in good quantity, we will try to crystallize the protein by using the Hampton screening reagents and the Emerald Biostructure kits. We will try the high throughput screening too. All the crystallization trials will be done at 4°C.

## **KEY RESEARCH ACCOMPLISHMENTS**

- Overexpression, purification and stabilization of the RIZ1 protein
- Preparation of a stable RIZ-PR domain sample for NMR experiments

## **REPORTABLE OUTCOMES**

None

## **CONCLUSIONS**

During the past year, we have successfully engineered and stabilized a third PR domain belonging to the same family of protein : the RIZ1 protein (325 amino acids). This protein will be introduced to crystallization trials which will allow us to determine its 3D structure.

We have also prepared a stable RIZ1-PR domain sample that we are using for NMR studies.

We are designing a new construct of the BLPR domain which hopefully will lead to success in its structural studies by crystallography.



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